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Evolution of Developmental Control Mechanisms

Genetic patterning in the adult capitate antenna of the beetle *Tribolium castaneum*

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ABSTRACT

Antenna structure varies widely among insects, in contrast to the well-conserved structure of legs. The adult capitate antenna of the red flour beetle, *Tribolium castaneum*, is composed of eleven articles, organized into four distinct morphological regions (scape, pedicel, funicle and club). Here, we report the use of RNA interference to examine the functions of 21 genes during antenna metamorphosis in *T. castaneum*. Genes with conserved functions relative to the developmental model species *Drosophila melanogaster* include *Distal-less* and EGF signaling (antennal growth), *spineless* (determination of antennal identity) and the Notch signaling pathway (antennal growth, joint formation, and sensory bristle development). However, the functions of many genes differed from those predicted from the *Drosophila* model. In addition to a conserved gap phenotype, depletion of *dachshund* transformed funicle articles toward club-like identity. Depletion of *Distal-less* or *homothorax* did not cause antenna-to-leg transformation. *Lim1* was required only for development of the scape-pedicle joint. Depletion of *odd-skipped*-related genes led to the loss of the entire funicle, while *spalt*, *rotund*, *spineless*, and *dachshund* affected smaller regions. Growth and joint formation were linked developmentally in the funicle, but not in the club. Joint formation within the club required *bric-a-brac*, *aristaless*, *apterous*, and *pdm*. Gene functions are discussed in terms of a model of antenna development in *T. castaneum*. This model provides a contrast to knowledge of antenna development in *D. melanogaster*, insight into the likely ancestral mode of antenna development, and a framework for considering diverse antenna morphologies.

Introduction

The antennae are sensory structures that house organs of hearing, olfaction, and tactile perception and are therefore crucial to the ability of insects to interact with their environment (Chapman, 1998; Snodgrass, 1935). All insect antennae possess three primary segments, the scape, pedicel, and flagellum. The flagellum is further subdivided into numerous articles in most adult insects. Like the primary segments, flagellar articles are separated by ectodermal joints, but they lack individual musculature (Snodgrass, 1928). Within this conserved structure, antenna morphology varies greatly among insect groups. In the fruit fly *Drosophila melanogaster*, the flagellum consists of four articles, the terminal of which is modified into an arista (Fig. 1D); this aristate morphology is common only among Diptera. Despite being the most speciose order of insects, most beetles (Coleoptera) share a relatively conserved antennal structure with nine flagellar articles (Minelli, 2005). Diversity of coleopteran antennae is achieved in large part by distinct patterns of regionalization within the flagellum. In the capitate antenna of the red flour beetle *Tribolium castaneum* (Herbst, 1797) the distal three articles are enlarged into a club, while the smaller intermediate articles are termed the funicle (Figs. 1A–C). Like most holometabolous insects, but not *Drosophila*, larvae of *T. castaneum* possess reduced antennae. In *Tribolium*, the larval antenna possesses three segments; homology between these and the adult antennal articles remains ambiguous (Švácha, 1992). During metamorphosis, growth and patterning transform this structure into the fully annulated adult antenna.

Studies of imaginal disc development in *D. melanogaster* (reviewed by Kojima, 2004) have provided a model for comparative studies of appendage development (Figs. 2A, B). The proximal-distal (PD) axis of imaginal discs that give rise to the ventral appendages is established by a gradient of secreted signaling molecules (Díaz-Benjumea et al., 1994; Lecuit and Cohen, 1997). This positional information is translated into a series of broad domains along the PD axis characterized by the expression of the transcription factors *homothorax* (*hth*) (Casares and Mann, 2001; Wu and Cohen, 1999), *dachshund* (*dac*) (Mardon et al., 1994), and *Distal-less* (*Dll*) (Cohen and Jürgens, 1989; Lecuit and Cohen, 1997). These genes act to promote both cell proliferation and regional identity. Loss-of-function mutations in these genes cause reduction or deletion of the limb regions in which they are normally expressed. This is analogous to the role that gap genes play in patterning the embryonic germband, lending these genes the name 'limb gap genes' (Rauskolb, 2001). *Dll* and a distal source of EGF signaling activate genes specific to distal appendage regions in *D. melanogaster* (Campbell and Tomlinson, 1998; Chu et al.,

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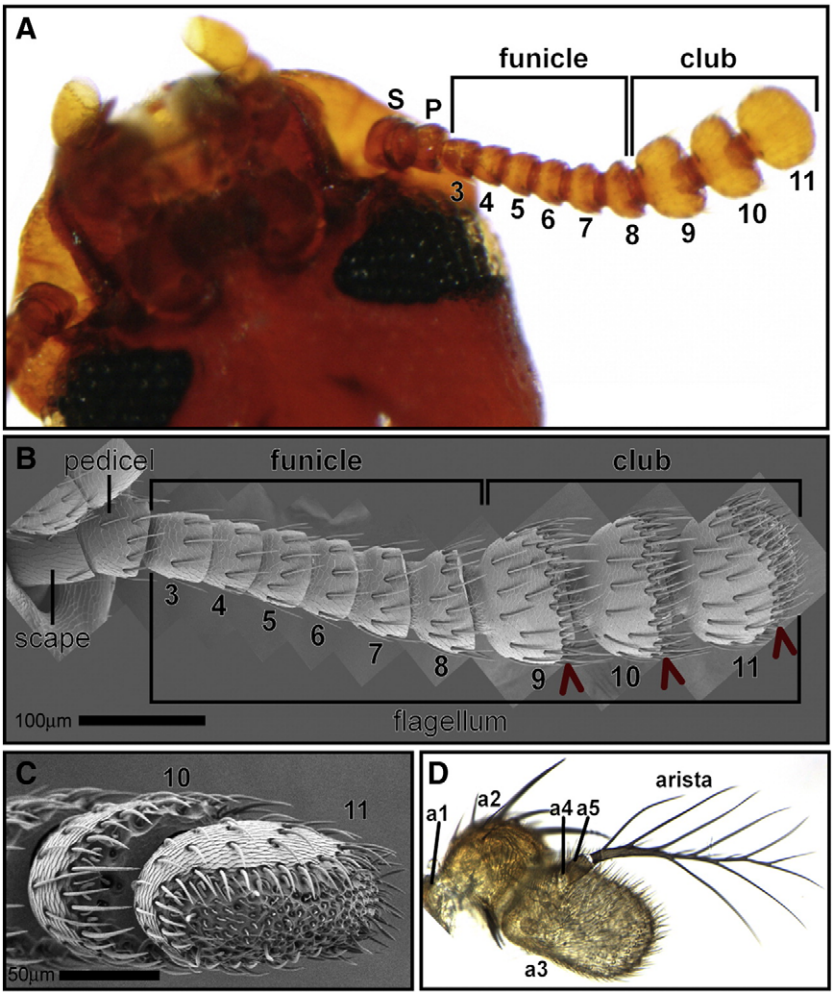

Fig. 1. Wildtype capitate morphology of the adult *Tribolium castaneum* antenna. (A) Light micrograph of the adult head and antenna in ventral aspect. (B) Scanning electron micrograph of the adult antenna in ventral view. Arrowheads indicate the row of distal macrochetes on club articles. (C) Scanning electron micrograph of the distal tip of the antenna. (D) The aristate antenna of *Drosophila melanogaster*. Abbreviations: S, scape; P, pedicel; flagellar articles are numbered 3 to 11; a1–a5, following *Drosophila* nomenclature, these indicate antennal article 1–5; a1 and a2 are the scape and pedicle, respectively.

2002; Duncan et al., 1998; Galindo et al., 2002; Tsuji et al., 2000). By the middle of the third instar, the presumptive flagellar articles and tarsi become distinguished by the expression and interactions of specific genes (reviewed by Kojima, 2004). Through interactions with *dac*, *Bar*, members of the *odd-skipped* (*odd*) gene family, and *spineless* (*ss*), expression of duplicate transcription factors at the *bric-a-brac* (*bab*) locus is refined into a series of rings (Chu et al., 2002; de Celis Ibeas and Bray, 2003). Proper *bab* expression is necessary for tarsal and distal antennal segmentation (Chu et al., 2002; Couderc et al., 2002; Godt et al., 1993; Rauskolb, 2001). Ultimately, joint formation is activated through Notch signaling just proximal to each presumptive joint location (Bishop et al., 1999; de Celis et al., 1998; Rauskolb, 2001; Rauskolb and Irvine, 1999).

Antennae are serially homologous to legs, and their development shares many similarities (reviewed by Angelini and Kaufman, 2005); however, several unique genes and interactions have been identified in the antenna of *D. melanogaster*. In the absence of Hox activity, *Dll* and *hth* overlap broadly in expression and cooperatively activate expression of *ss*, which specifies antennal identity by regulating downstream targets (Dong et al., 2000; Emerald et al., 2003; Emmons et al., 2007). Dipterans are derived in many aspects of appendage development, and comparative developmental genetic studies have suggested that the gene functions and interactions involved in *Drosophila* appendage patterning are a mixture of derived and ancestral ones (reviewed by Angelini and Kaufman, 2005; Ober and Jockusch, 2006). Therefore, models based on appendage patterning of *D. melanogaster* do not clearly predict how morphologically distinct appendages in other insect groups might be produced. For example, it is unknown how antennal identity is specified in *Tribolium*, or what genes direct growth and patterning of the morphologically distinct regions of the flagellum.

To address these questions, we conducted an RNA interference (RNAi) screen of candidate genes for adult antennal patterning in *T. castaneum*. While some genes (e.g., *ss*, *Serrate* (*Ser*), and an EGF ligand) produce depletion phenotypes similar to their loss-of-function phenotypes in *D. melanogaster*, many RNAi depletions reveal functions unlike the role of orthologues in *Drosophila* appendage development. These data provide a genetic model for antenna development in a species with the common and phylogenetically widespread capitate morphology of many Coleoptera.

Materials and methods

Beetle handling

Cultures were established from wildtype *Tribolium castaneum* adults obtained from Carolina Biological Supply Company. Beetles were cultured under standard conditions (Sokoloff, 1972) with the addition of the antifungal compound Fumagilin (Walter T. Kelly, Co.) at 1:40,000 w/w.

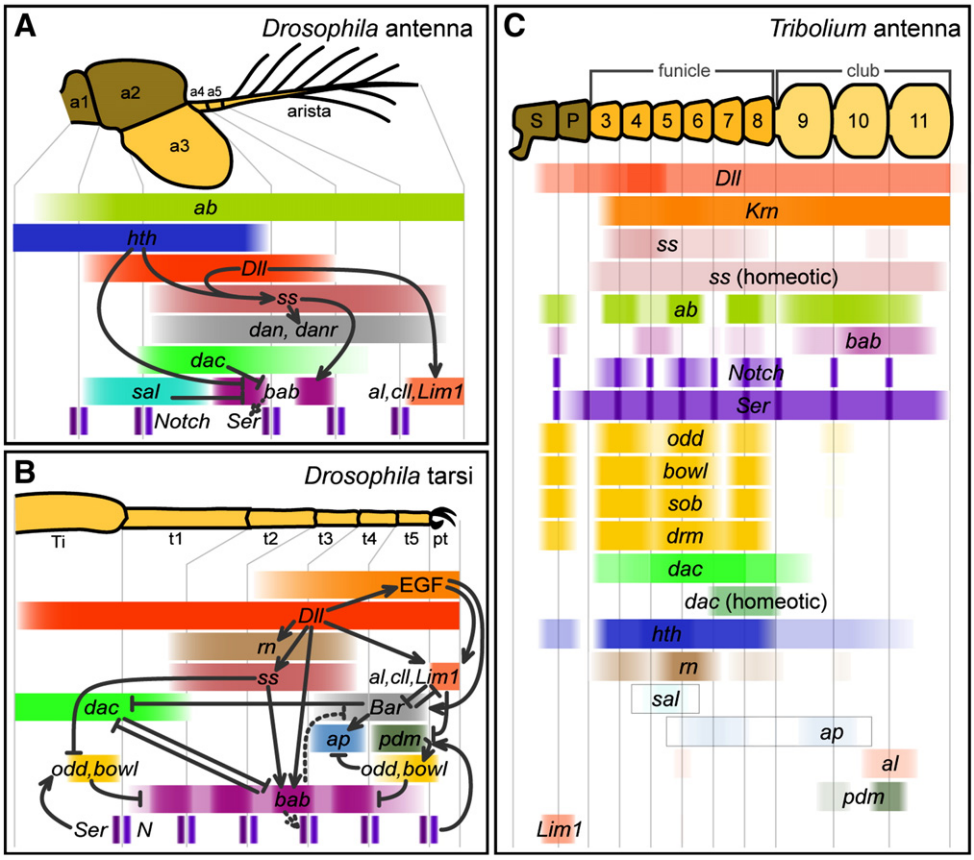

Fig. 2. Patterning of the antenna and distal leg in *Drosophila* and *Tribolium*. Models of genetic interactions in the antennal (A) and distal leg (B) third instar imaginal discs of *D. melanogaster* provided the starting point for selection of candidate genes. (See text for references.) The regions of gene expression are mapped onto the adult appendage structures shown above. Gene interactions are indicated by the black lines: activation is denoted by pointed arrows, repression by blunt-ended lines. Hypothesized interactions are shown as dashed lines. (C) Summary of the regions of the *T. castaneum* antenna affected by RNA interference of specific genes. The opacity of colored bars indicates the penetrance of particular dsRNA treatments in the corresponding region of the adult antenna diagrammed above.

Isolation and cloning of candidate gene sequences

Candidate genes (Table 1) were selected based on known functions in the patterning of *D. melanogaster* antennae or distal legs. Orthologues in *T. castaneum* were identified by tBLASTn search against the genome sequence (Tribolium Genome Sequencing Consortium, 2008; http://www.ncbi.nlm.nih.gov/projects/genome/guide/beetle/). Orthology was confirmed by reciprocal search of the highest scoring

Table 1
A list of candidate genes examined in this study

Gene		Protein class	LG	GenBank	Clone source
abrupt	*ab*	BTB/Zn-finger TXF	5	XM_969854	This study
aristaless	*al*	Homeobox TXF	5	NM_001114366	This study
apterous-related	*ap1*	Homeobox TXF	3	XM_965020	Y. Tomoyasu, unpublished
	ap2	Homeobox TXF	3	XM_001810931	Y. Tomoyasu, unpublished
bric-a-brac	*bab*	BTB/Psq TXF	3	XM_001812888	This study
clawless/C15	*cll*	Homeobox TXF	9	XM_969890	This study
dachshund	*dac*	Novel TXF	4	XM_964678	Prpic et al. (2001)
Distal-less	*Dll*	Homeobox TXF	7	NM_001039439	Beermann et al. (2001)
homothorax	*hth*	Homeobox TXF	7	NM_001039400	Angelini & Kaufman (2004)
Keren	*Krn*	EGF ligand	3	XM_001813564	This study
Lim1	*Lim1*	Homeobox TXF	6	XM_964391	This study
Notch	*N*	Notch receptor	10	NM_001114381	This study
odd-skipped	*odd*	Zn-finger TXF	8	XM_966993	This study
brother of odd with entrails limited	*bowl*	Zn-finger TXF	8	XM_967045	This study
sister of odd and bowl	*sob*	Zn-finger TXF	8	XM_966942	This study
drumstick	*drm*	Zn-finger TXF	8	XM_966887	This study
pdm/nubbin	*pdm*	Homeobox TXF	4	XM_963346	This study
rotund	*rn*	Zn-finger TXF	?	XM_966094	This study
spalt	*sal*	Zn-finger TXF	5	XM_968136	Tomoyasu et al. (2005)
Serrate	*Ser*	Dl/Ser-type EGF	7	XM_964393	This study
spineless	*ss*	bHLH/PAS TXF	10	XM_962783	This study

The chromosomal linkage group (LG) is listed, as is the GenBank accession number of the known or predicted transcript. Abbreviations: bHLH, basic helix-loop-helix domain; BTB, Bric-a-brac/Tramtrak/Broad complex domain; EGF, epidermal growth factor; PAS, PER-ARNT-SIM domain; Psq, pipsqueak; and TXF, transcription factor.

results against the *D. melanogaster* genome. Fragments (180–800 bp) from each target gene were amplified from pupal cDNA, cloned into the Topo4-TA vector (Invitrogen), and sequenced to confirm identity.

Relative real-time PCR

The relative expression levels of genes in the club and proximal antenna were determined using real-time PCR. Roughly 40 pupal antennae were dissected into club and proximal regions, and immediately transferred into Trizol (Invitrogen) for RNA extraction. Reverse transcription was performed using the iScript cDNA Synthesis Kit (BioRad). Amplification of target cDNA was detected using a SYBR Green polymerase mix (Absolute Scientific) on an iCycler instrument (BioRad). Relative target sequence abundance was calculated using the method described by Pfaffl (2001) with *18S* ribosomal RNA as a reference. All primer sequences are available from the authors upon request.

RNA interference

RNA interference (RNAi) was used to examine depletion phenotypes in adult beetles following the procedures of Tomoyasu and Denell (2004). Template DNA for double-stranded RNA (dsRNA) transcription was produced by amplification from cloned candidate gene fragments using primers including a 5′ T7 promoter sequence. T7 RNA polymerase was then used to simultaneously synthesize RNA from both DNA strands. Following annealing and purification, dsRNA was diluted to 1 μg/μl in saline buffer. Approximately 160 nl dsRNA was injected into the dorsal prothorax of prepupal larvae using a pulled-glass capillary needle. For some dsRNAs, this concentration caused high lethality, in which case we repeated injections with a 1:10 or 1:100 dilution. Multi-gene RNAi treatments combined equal masses of each dsRNA. Table 2 lists dsRNA size, survival rate and penetrance for each RNAi treatment. To look for potential off-target effects of RNAi, sequence matches for dsRNAs were examined using BLAST against the *T. castaneum* genome sequence. In *D. melanogaster*, it appears that regions of nucleotide identity longer than 16 bp are required (but not sufficient) for off-target effects (Kulkarni et al., 2006). Exact matches exceeding 16 bp in length are rare in the *T. castaneum* genome (Suppl. table 1). This, combined with the expectation that the genes examined would have appendage phenotypes, suggests that off-target effects are unlikely to be the cause of the observed appendage-specific phenotypes.

Each surviving individual was scored as wildtype or abnormal at each antennal segment and joint. This character matrix was used to calculate overall phenotypic penetrance and penetrance in each article of the antenna.

Table 2
The frequency of results from larval RNA interference

dsRNA	Size (bp)	Number injected	Survived to metamorphosis	Phenotypic penetrance	
				Appendages	Antennae
Buffer	–	120	37	0[a]	0
ab	198	45	10	70%	70%
al	172	86	31	39%	8%
ap1	508	53	22	5%	0
ap2	514	36	5	0	0
bab	774	42	22	82%	59%
cll	171	129	54	0	0
dac	359	130	15	73%	73%
Dll	462	150	39	90%	74%
hth	335	50	10	70%	70%
Krn	188	40	8	100%	94%
Lim1	388	27	13	92%	62%
Notch	409	90	11	100%	100%
odd	211	42	9	100%	78%
bowl	342	24	11	91%	86%
sob	321	27	16	94%	91%
drm	225	58	9	89%	78%
pdm	332	113	24	33%	25%
rn	127	51	19	63%	45%
sal	200	70	24	29%	8%
Ser	329	43	7	100%	93%
ss	355	35	20	95%	68%
ap1-2	–	56	36	14%	8%
al, Lim1	–	27	10	20%	15%
al, cll, Lim1	–	16	8	75%	69%
odd, bowl, sob	–	58	7	100%	29%
odd, bowl, sob, drm	–	40	13	92%	85%
overall		1658	30%	66%	53%

The size of dsRNAs and the number of injected individuals are provided. Individuals surviving to metamorphosis were categorized as wildtype if the external morphology of the ventral appendages (antennae, mouthparts, legs, and female genital styli) did not deviate from that of buffer-injected individuals. Phenotypic penetrance is given as the percentage of surviving individuals that display a ventral appendage phenotype. Penetrance values are shown for the overall external anatomy, as well as for the antennae. Single and multiple dsRNA treatments are listed separately, with average survival and penetrance for each group.

[a] Rarely buffer-injected, control individuals were recovered with a single leg truncated to the coxa or entirely absent (n=3 of 120). This defect is likely to be an artifact of the injection method, and its occurrence in experimental treatments was not counted as a phenotype (n=15 of 1658).

Microscopy and imaging

The morphology of adult beetle cuticles was examined after clearing in 20% glycerol in glacial acetic acid (modified from Van der Meer, 1977). Specimens were then washed into 70% glycerol and stored at −20 °C before dissection and imaging. Light micrographs of dissected antennae were obtained with an Olympus digital camera on a Zeiss Axioskop compound microscope. Some specimens were prepared for electron microscopy by overnight dehydration in ethanol, followed by immersion in hexamethyldisilazane for 15 min. Gold-palladium coated specimens were then imaged using a Zeiss DSM982 Gemini field emission scanning electron microscope.

Results

Isolation and characterization of candidate genes

We searched for orthologues of a variety of candidate genes for antenna development (see Figs. 2A, B) in the genome of *T. castaneum*. The gene *apterous* exists in tandem duplication in *T. castaneum*, and a single ORF is orthologous to *Drosophila bab1* and *bab2*. Four *odd*-skipped family members are found in both *T. castaneum* and *D. melanogaster*. Reciprocal BLASTp searches identified three pairs that appeared orthologous; however, for *T. castaneum odd-skipped* (*odd*), the highest scoring match in *D. melanogaster* was the *odd* family member *brother of odd with entrails limited* (*bowl*). Thus, orthology of the *odd-skipped* gene family was further examined by phylogenetic analysis (Suppl. fig. 1). The resulting group of 21 candidates was cloned for expression and functional study (Table 1).

Results of relative real-time PCR analyses are summarized in Fig. 3. The relative level of transcript abundance varied across nearly five orders of magnitude (Fig. 3A) but did not correlate with gene function; nor did it have a significant correlation with the penetrance of RNAi effects (not shown). Comparison of club and proximal (scape, pedicle and funicle) cDNA pools demonstrated that several genes were significantly enriched in one of these regions (Fig. 3B). In general, there was agreement between regions affected by RNAi depletion of a gene and the region of enrichment determined by real-time PCR.

RNA interference produced a range of phenotypic effects for most genes that could be ordered from mild to severe, based on the size of the affected region, as with a hypomorphic series. While survival was sometimes low (averaging 30%; Table 2) phenotypes were typically highly penetrant (averaging 55%) and consistent across individuals injected with the same dsRNA. Only a single treatment, RNAi against

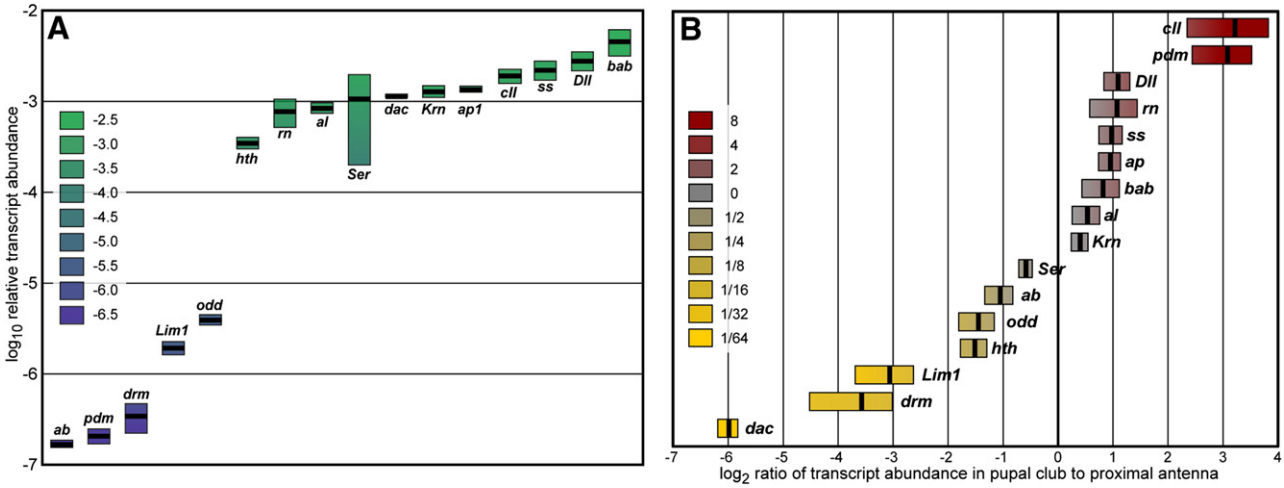

Fig. 3. (A) Abundance of candidate transcripts as determined by relative real-time PCR. The scale shows expression levels relative to *18S* ribosomal RNA on a $\log_{10}$ scale. (B) Expression ratio of candidate transcripts in funicle and proximal antenna (left) versus club (right). Fold-abundance in the club is shown on the horizontal axis on a $\log_2$ scale. In both panels, the mean ratio is denoted by the heavy line. Boxes indicate 1 standard deviation from the mean.

cll, failed to yield phenotypic individuals (Table 2). These tests of gene function reveal several distinct processes involved in the development of the *T. castaneum* antenna. In Fig. 2C RNAi effects along the PD axis of the antenna are summarized for each gene.

Growth and elongation along the PD axis are promoted by Keren, Serrate, and Distal-less

RNA interference against three genes led to severe reduction in the length of the flagellum, indicating that these gene products act to promote growth and elongation along the PD axis. RNA interference against *Keren* (*Krn*), the only activating EGF ligand present in the *T. castaneum* genome (Tribolium Genome Sequencing Consortium, 2008), lead to a drastic reduction in the length of the flagellum, but the scape and pedicel were relatively unaffected (Fig. 4B; compare to Fig. 4A). Reduction in length was accompanied by reduction in the number of flagellar articles, and in the most severely affected *Krn* RNAi phenotypes all joints were lost from the flagellum. Similarly, the most severe depletion phenotype for *Ser*, a ligand that activates the Notch signaling pathway, was a strong reduction in antenna length and the complete absence of joints (Fig. 4C). Phenotypes generated by RNAi against *Dll* closely resembled those seen in *T. castaneum Fused tarsi and antenna* mutants (Fig. 4D), which carry hypomorphic alleles at the *Dll* locus (Beermann et al., 2001), as well as appendage phenotypes resulting from *Dll* depletion in other arthropods (Angelini and Kaufman, 2004; Schoppmeier and Damen, 2001). In moderate phenotypes the length of the flagellum was reduced, and fewer articles were present (Fig. 4E). In severely affected *Dll* resultants the antenna was greatly reduced and joints failed to form. Surprisingly, in some mild *Dll* phenotypes (7 of 58 non-wildtype resultant antennae), a twelfth article appeared at the distal tip, possibly as a result of ectopic joint formation within the terminal article. This was the only gene whose depletion caused an increase in the number of articles. Even in the most severely affected *Krn*, *Ser* and *Dll* resultants, antennae were wider distally than proximally, indicating the presence of both funicle and club regions (red line in Figs. 4–6).

To a lesser degree the gene *abrupt* (*ab*), which encodes a zinc finger transcription factor, also affects growth and elongation of the PD axis. In strongly affected individuals, a reduction in length and fusions of adjacent segments occurred in all regions of the antenna (Fig. 4F). Mildly affected individuals showed fusion only of funicle articles 5–6. Three club articles were identifiable in all individuals, but the number of funicle articles was reduced to as few as two, concomitantly with a great reduction in the length of the funicle.

Joint formation requires activation of the Notch signaling pathway

The differentiation of joints between segments and articles is a process that requires activation of the Notch signaling pathway. In *T. castaneum* RNA interference against *Notch* led to the absence of most antennal joints (Fig. 4G). This phenotype is distinct from the fusion of articles caused by other RNAi treatments because *Notch* depletion did not lead to a substantial reduction of growth along the PD axis. The cuticle of *Notch*-depleted individuals lacked most of the bristles normally present. However, the ring of macrochetes indicating the distal edge of all three club articles remained unaffected in *Notch* RNAi resultants, suggesting that despite the absence of joints, regions of funicle and club identity, and a full complement of club articles, persist. Interestingly, the pedicel-flagellum joint remained in all *Notch* knockdown individuals.

RNA interference against *Serrate*, a Notch ligand, produced similar effects on joint development (Fig. 4C). Mildly affected resultants displayed an absence of the scape-pedicel joint. In more strongly affected individuals flagellar joints were also absent, although individual articles were still recognizable based on bristle patterns. However, unlike *Notch* RNAi, depletion of *Ser* activity did not reduce the density of bristles on the antenna. The pedicel-flagellum joint was relatively robust to depletion, but was eliminated in strong *Ser* RNAi phenotypes, which were greatly reduced in length and lacked all joints. The pedicel-flagellum joint corresponds to the coxopodite-telopodite boundary (Snodgrass, 1935) and may develop through unique mechanisms.

Antennal identity in the flagellum is specified by spineless, but does not require Distal-less or homothorax at metamorphosis

We examined the effects of depletion of orthologues of three transcription factors that cause antenna-to-leg transformations in *D. melanogaster* (Dong et al., 2000). In *T. castaneum*, depletion of *ss* resulted in homeotic transformations, while depletion of *Dll* or *hth* did not. Depletion of *ss* using RNAi in *T. castaneum* produced phenotypes ranging in severity from mild fusion of funicle articles to transformation of the flagellum toward leg structures (Fig. 5A). Often the antenna was reduced in length and number of joints in both the funicle and club, but also possessed a distal claw. In more severely affected individuals, the funicle and club regions resembled the tibia in shape and bristle pattern. These transformed appendages had a claw joined distally to a tibia-like structure–no tarsal structures were apparent–unlike *Drosophila ss* mutations in which distal antenna structures are

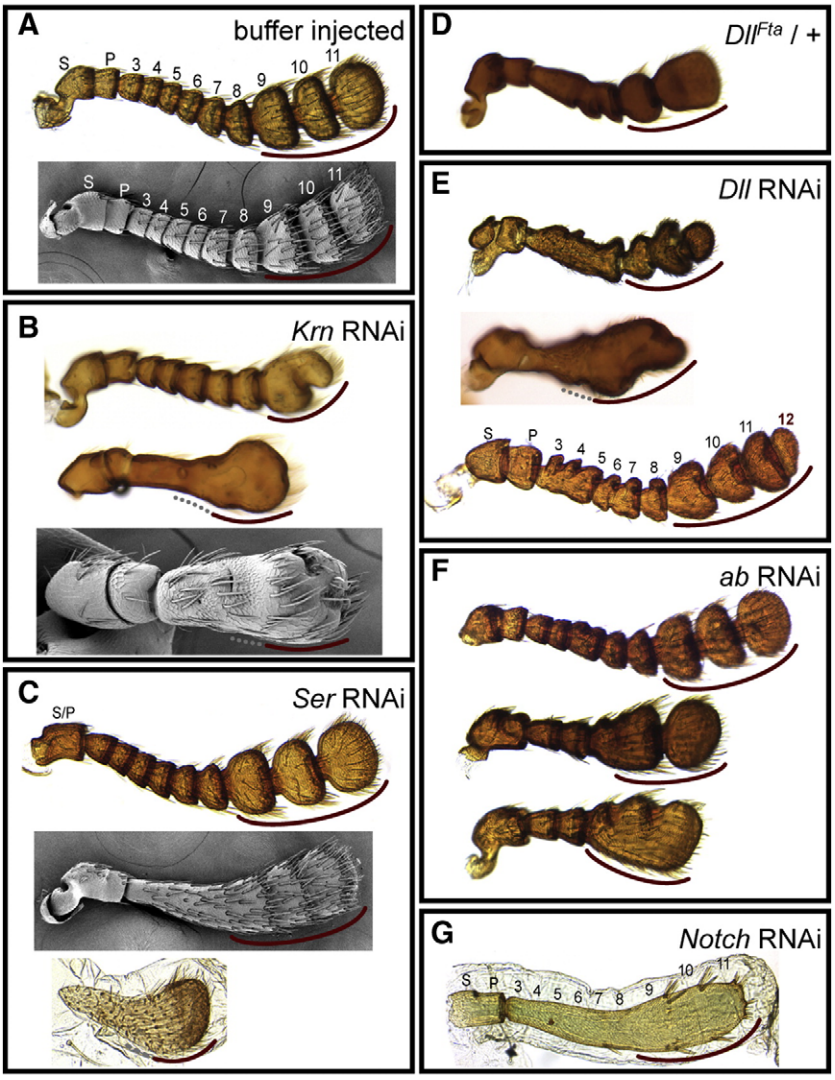

Fig. 4. Results of RNA interference producing defects primarily in antenna length or joint formation. All antennae are oriented with the proximal point of attachment to the left and the ventral side toward the top of the page. The wildtype *T. castaneum* antenna includes 11 articles: the scape, pedicel, and nine articles of the flagellum. (A) Buffer injected individuals display wildtype morphology. Articles 9–11 form the club, in which articles are enlarged and bear a distal ring of macrochetes, visible in scanning electron micrographs (SEM). A red line denotes the club in each panel. A dashed gray line is used where identity is uncertain. (B) *Krn* RNAi reduced antenna length and number of articles. However, the scape and pedicel were relatively unaffected. (C) Depletion of the ligand encoded by *Ser* caused a failure of joint formation and reduction in antenna length. Mild phenotypes showed fusion of the scape/pedicel joint. Strong *Ser* phenotypes had a drastic reduction in antenna length and lacked all joints. (D) A hypomorphic allele of *Dll* known as *Fused tarsi and antennae* (*Fta*) produces reduced antenna length and article number. (E) Similar effects were found in *Dll* RNAi. Strongly affected individuals had a complete fusion of the flagellum and severe reduction in size. Mildly affected specimens had poorly formed joints and a "serrated" appearance; rarely an extra distal article appeared. (F) Depletion of the gene *ab* resulted in fusion of adjacent articles throughout the antenna. Moderate reduction in antennal length was caused primarily by shortening of the funicle. (G) *Notch* RNAi produced phenotypes lacking all joints except the pedicel-flagellum joint. In addition, most of the bristles were lost from the cuticle, except for the macrochetes that indicate the distal edges of club articles. Abbreviations as in Fig. 1.

transformed into tarsus and claw. The likelihood of homeotic transformation depended on the timing of injection in *T. castaneum*; specimens injected shortly before pupation showed fusions in the flagellum, but not transformation, suggesting that *ss* has distinct roles at different developmental stages (Suppl. Fig. 2).

Specification of regional identity within the funicle requires dac activity

Transformations of identity within the antenna occurred between flagellar regions as a result of depletion of *dac*. In many *dac* RNAi resultants, the distal two articles of the funicle (7 and 8 in wildtype, see Fig. 2C) displayed a homeotic transformation toward club-like identity (Fig. 5B). These articles were larger in width and length than in the wildtype funicle and had bristle patterns indicative of club identity, such as the distal ring of macrochetes (Fig. 5C). Depletion of *dac* also caused substantial reductions in the funicle. Specimens with a greater degree of transformation toward club appeared to have more reduced proximal funicle articles, and the distal-most funicle article was sometimes fused to the adjacent club article. These effects demonstrate that *dac* acts as a gap gene to promote growth of the funicle, but also as a homeotic gene in articles 7 and 8, where it directs funicle identity or prevents differentiation as club. By contrast, the transformation of club articles toward funicle identity was not observed for any gene.

odd-skipped family genes act as gap genes in funicle development

The *T. castaneum* genome includes four members of the *odd-skipped* family, *odd*, *bowl*, *sister of odd and bowl* (*sob*), and *drumstick* (*drm*) (Suppl. fig. 1). Phenotypes resulting from injection of dsRNA from individual *odd*-related genes were similar (Figs. 5D–G). In mildly affected individuals, articles of the funicle were fused at odd-

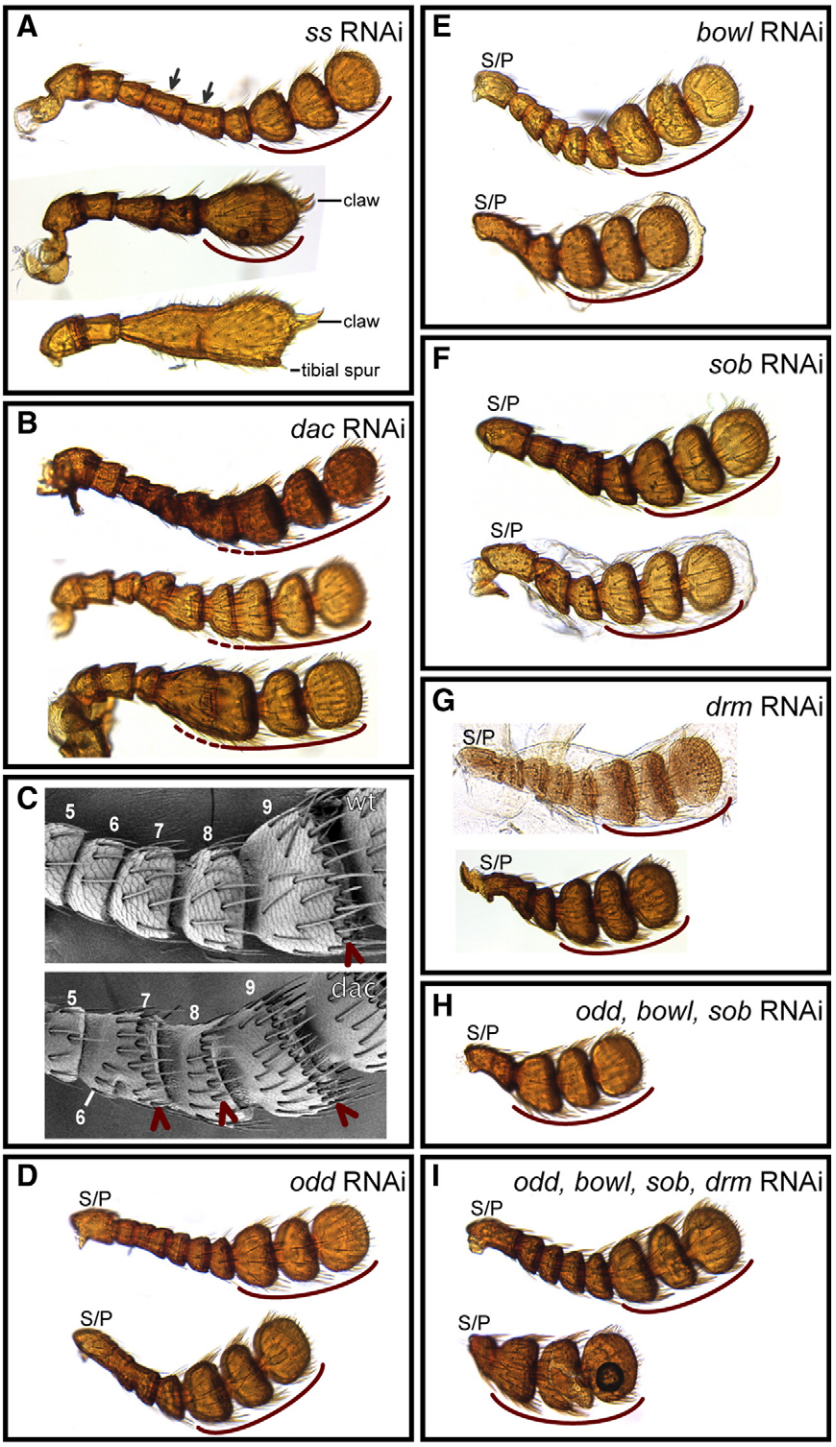

Fig. 5. Results of RNA interference producing homeotic defects (A–C) or gap defects in the funicle (D–I). All panels are oriented and labeled as in Fig. 4. (A) RNAi against *ss* caused fusion of funicle articles (arrows) without substantial reduction in funicle length. More strongly affected individuals had a homeotic transformation of the distal flagellum toward a leg-like identity, as indicated by the presence of a distal claw, tibial spur, and tibia-like bristle pattern. The scape and pedicel remained unaffected. (B) Depletion of *dac* activity by RNAi caused reduction of the funicle and fusion of articles in this region, as well as homeotic transformation of the distal funicle articles toward a club-like identity (dashed red line). (C) SEM of articles 6 to 9 from wildtype and a *dac* RNAi resultant. In the wildtype antenna, club articles are enlarged and the distal edge is ringed with macrochetes (red arrowhead). In the *dac* RNAi antenna, articles 7 and 8 were also enlarged and carried ectopic distal macrochetes (red arrowheads). (D–G) RNA interference against *odd*-related genes caused fusions and reduction in the funicle and fusion of the scape and pedicel. (H–I) Combined dsRNA pools targeting *odd*, *bowl*, and *sob* or these genes and *drm* caused the complete deletion of the funicle. Abbreviations as in Fig. 1.

even joints and the funicle was reduced in overall size. The funicle was reduced to a single article in the most severe single-gene treatments (Fig. 5F). Scape-pedicel fusions also occurred at high frequency. Rarely, in severely affected specimens, the proximal club articles were also fused (5 of 66 antennae). The dsRNA constructs used for *odd*, *bowl*, and *sob* (but not *drm*) targeted regions of high sequence similarity across these paralogues (Suppl. table 1). Thus, one explanation for the similarity in knockdown phenotypes is that multiple genes were depleted by a single dsRNA. As a result, specific functions cannot be attributed to each paralogue. To test explicitly for redundant functions, we also injected individuals with equal masses of dsRNA from *odd*, *bowl*, and *sob* (Fig. 5H) or *odd*, *bowl*,

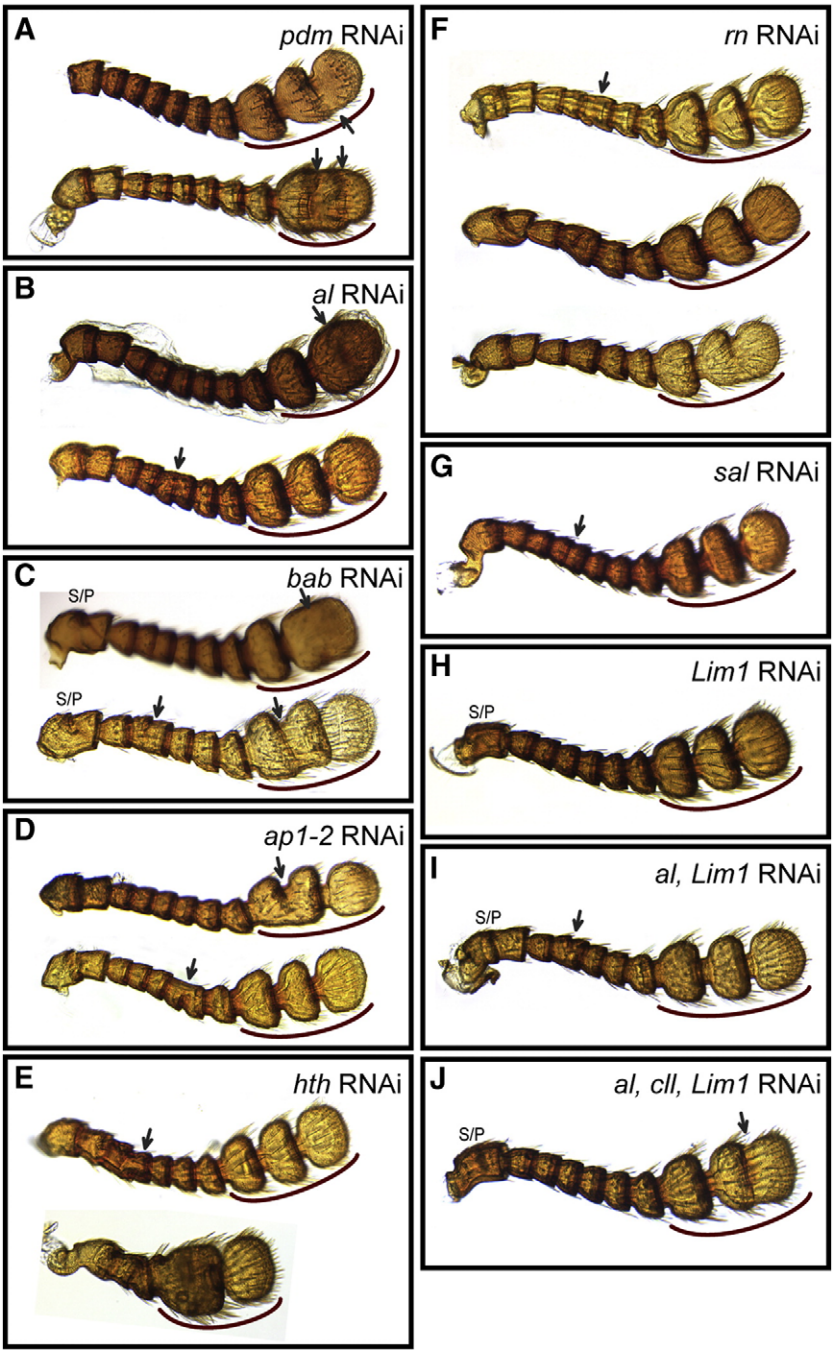

Fig. 6. Results of RNA interference producing defects in local regions. All panels are oriented and labeled as in Fig. 4. Arrows indicate joint loss or fusion in mild phenotypes. (A) Fusion of two or three club articles resulted from *pdm* RNAi. (B) Depletion of *al* activity resulted in fusion of the distal articles, 10 and 11. Rarely articles 5 and 6 were fused. (C) RNAi against *bab* caused fusions in the club and between the scape and pedicel. In some individuals fusions also occurred in the funicle. (D) Combined RNAi knockdown of *ap1* and *ap2* caused fusion within the club, or rarely the partial fusion of adjacent funicle articles. (E) Depletion of *hth* caused reduction and fusion within the funicle. Severely affected individuals sometimes also showed fusion of the proximal club article. (F) Depletion of *rn* typically caused fusion and reduction of the number of articles in the funicle. Occasionally, club articles also were fused. (G) Articles 4–5 of the funicle were fused in *sal* RNAi. (H). *Lim1* RNAi caused the fusion of the scape and pedicel, but no defects in the flagellum. To test for potential interactions between Lim1 and distal genes known to interact with it in *D. melanogaster*, we examined *al*, *Lim1* (I) and *al*, *cll*, *Lim1* (J) RNAi combinations. These individuals had scape-pedicle fusions, as in *Lim1* RNAi, and fusions within the club, as in *al* RNAi. Note that *cll* RNAi produced no detectible phenotype (see Table 2). Abbreviations as in Fig. 1.

sob, and *drm* (Fig. 5I). These multi-gene treatments yielded phenotypes similar to those from single gene depletions, as well as more severe phenotypes in which the funicle was entirely deleted, such that an apparently wildtype club was attached distal of a fused scape and pedicel (Figs. 5H, I). Interestingly, the *odd*-related genes are the only genes examined here for which RNAi produced the complete deletion of an antennal region without also having drastic effects on the morphology of the remaining structures.

Within local areas distinct genes promote growth or maintenance of cells and joint formation

Fusion or loss of segments was one of the most common phenotypes observed in RNAi resultants, but most genes had specific regions of effect. This suggests that Notch-mediated joint formation may be activated locally by different factors. Interestingly, these local effects are often restricted to particular anatomical regions (club, funicle, or coxopodite).

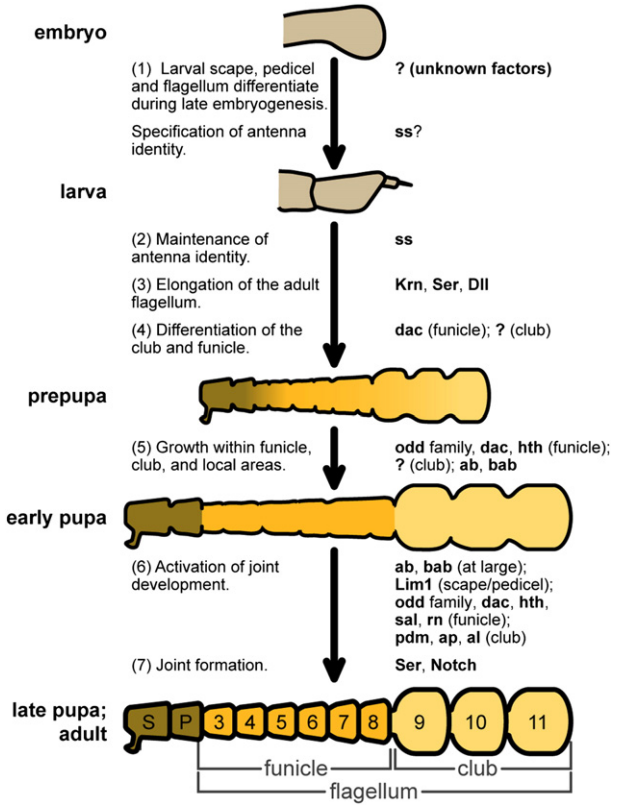

Fig. 7. A model for development of the *T. castaneum* antenna. At each developmental stage ontogenetic events are described on the left; genes required for these processes are listed on the right. Coloration denotes the differentiation of scape/pedicel, funicle and club. Homology between larval and adult regions is uncertain.

Fusions of club segments were caused predominantly by dsRNA targeting *POU-domain protein* (*pdm*), *aristaless* (*al*), *apterous* (*ap*) paralogues, and *bric-a-brac* (*bab*). In all cases, three distinct club segments could still be identified, and there was no substantial effect on growth within the club. RNA interference against *pdm* (Fig. 6A), *al* (Fig. 6B) or *bab* (Fig. 6C) generally caused fusions of the distal club articles (10–11). Fusion of the entire club was also found for *pdm* and *bab*. The genome of *T. castaneum* includes two tandem paralogues of *apterous* (Table 1), referred to here as *ap1* and *ap2*. RNA interference against either paralogue alone yielded no antennal phenotype (Table 2). However, in RNAi using pooled *ap1* and *ap2* dsRNA, the proximal club articles (9–10) were fused (Fig. 6D). While the effects of *pdm* and *al* were confined to the club, *bab* and *ap1-2* resultants also sometimes showed fusions in the funicle. Conversely, *rotund* (*rn*) and *hth*, which predominantly caused reductions and fusions in the funicle, also caused fusions in the club at low frequency (Figs. 6E, F).

RNA interference revealed a function for several genes in the development of the funicle, including *dac* (Fig. 5B), *ss* (Fig. 5A), *hth* (Fig. 6E), *rn* (Fig. 6F), and *spalt* (*sal*; Fig. 6G). Unlike the *odd*-related genes, knockdown of these factors never produced the deletion of all funicle articles. The articles that were affected differed between genes, indicating that these genes have distinct roles in funicle patterning. In general, in mildly affected individuals, six funicle articles could still be identified, with some degree of fusion between articles. In more severely affected individuals, the funicle was reduced in length and the number of articles was decreased, with a greater degree of reduction observed for *hth* and *dac* than for *rn* and *sal*. An overall correlation between the length of the funicle and the number of articles, combined with a general tendency for articles that were individuated to resemble wildtype articles in size and shape, suggests that growth of the funicle and joint formation are developmentally linked. One exception to this pattern was in mildly affected *ss* individuals, in which the proximal and distal funicle articles differentiated normally, and were separated by two elongated articles interpreted as fusions of 4 to 5 and 6 to 7 (Fig. 5A).

One gene was determined to have a specific function limited to development of the proximal antenna. Depletion of *Lim1* by RNAi in *T. castaneum* caused loss of the joint between scape and pedicel (Fig. 6H) Real-time PCR data confirmed that expression of *Lim1* is highly enriched proximally (Fig. 3). Scape-pedicel fusions also commonly occurred as a result of depletion of *odd* family genes, and, less frequently, as a result of RNAi against *Dll*, *ab*, *bab* and *hth*. By contrast, loss of the pedicel-flagellum joint was rare, occurring only in the strongest phenotypes resulting from RNAi against *Dll* and *Ser*, in which all joints were eliminated.

Discussion

Genetic patterning of the Tribolium castaneum adult antenna

The antenna of *T. castaneum* represents a segmented structure with regions of distinct identity in which to explore developmental patterning. Fig. 2C summarizes the regions affected by RNAi experiments in this study. Functional tests of the 21 candidate genes reveal five distinct developmental functions: (1) Growth and elongation along the PD axis are promoted by the genes *Krn*, *Ser*, and *Dll*, and to a lesser degree by *ab*. (2) The differentiation of joints between segments and articles is a process that requires activation of the Notch signaling pathway. (3) The antennal identity of the flagellum is maintained by the activity of *ss* at metamorphosis. When *ss* is depleted by RNAi, transformation of the flagellum toward tibia and claw identity results. It is not known what factors identify the scape and pedicel as antennal structures. (4) Development of the funicle requires *odd* paralogues and *dac*. Depletion of *odd*-related genes leads to complete deletion of the funicle. The activity of *dac* is required for the proper identity of distal funicle articles, and in *dac* RNAi resultants, distal funicle articles are transformed toward club-like identity. (5) Growth or maintenance of cells within specific regions is accomplished by the local activity of different sets of genes. RNAi against genes including *odd*-related paralogues, *dac*, and *hth* causes large gap deletions within the funicle. The funicle is reduced to a lesser extent as a result of RNAi against *rn* or *sal*. Genes required for proper segmentation of the club include *ap*, *al*, *bab* and *pdm*, while depletion of *Lim1* leads specifically to the loss of the scape-pedicel joint. These results suggest that Notch-mediated joint formation is activated through different pathways in different regions.

Many of these developmental events appear to be genetically separable, since RNA interference against most genes results in only one class of defect. However, some genes appear to be involved in multiple processes, such as *dac* in the growth and identity of the funicle, *ss* in appendage identity and patterning of the funicle, and *Ser* in the growth of the early antenna and in joint formation.

A comparison of antennal patterning in Tribolium and Drosophila

In order to understand how genetic patterning produces anatomical structures, it is useful to consider the development of homologous structures in morphologically divergent species (Figs. 2A–C). The antennae of *Drosophila* and *Tribolium* share a jointed structure that is differentiated along the PD axis, but differ in the number of articles, the way in which articles are grouped into regions of like identity, and the specific structure of articles (Figs. 1A, D). We expected to find differences in the developmental system related to the distinct aristate morphology of *D. melanogaster* and the capitate morphology of *T. castaneum*, while still finding similarities inherited from the shared ancestral developmental program of the insect antenna.

The most interesting genes for understanding the origins of morphological differences are those with qualitatively different genetic functions or domains of effect. One of the clearest examples from this study is *Lim1*. In *D. melanogaster Lim1* functions in a feed-forward network with *al* and *clawless*/*C15* (*cll*) to promote identity and growth of the pretarsus and arista (Campbell, 2005; Tsuji et al., 2000). However, in *T. castaneum*, expression of *Lim1* is enriched proximally (Fig. 3B), and depletion of *Lim1* in *T. castaneum* produces fusion of the proximal-most segments, not defects in the distal antenna (Fig. 6H). Simultaneous introduction of dsRNAs for *al* and *Lim1* (or *al*, *cll*, and *Lim1*) resulted only in the additive combination of *al* and *Lim1* phenotypes (Figs. 6I, J). Therefore *Lim1* is unlikely to interact with *al* or *cll* in *T. castaneum*. These results show that the functions of *Lim1* orthologues have diverged along the lineages separating *T. castaneum* and *D. melanogaster*. Another example of divergent function comes from the *odd*-related paralogues, which were required for the development of the antennal funicle in *T. castaneum*, indicating that they function as antennal gap genes (Figs. 5A–K). By contrast, in *D. melanogaster* the *odd*-related genes have not been implicated in antenna development. Their role in tarsal development is as a modulator of other factors influencing tarsal growth and identity, such as *bab* (de Celis Ibeas and Bray, 2003), and loss of function in the *odd* paralogues does not produce the deletion of an entire region of similar identity, as seen in *T. castaneum* antennae.

Finally, another noteworthy difference between *Drosophila* and *Tribolium* is in the distinct roles of *dac* in antenna development. In *D. melanogaster*, mutations in *dac* have very limited effects on antennal development, despite a broad expression domain (Dong et al., 2002) *dac* is expressed primarily in the large first flagellar article of the developing antenna; however, elimination of *dac* activity leads only to the loss of the distal joint between the fifth article and the arista and a slight change in the morphology of a5. Antennal development is normal in hypomorphic *dac* mutants (Dong et al., 2002). By contrast, *dac* has two important functions in the antennal development of *T. castaneum*, as revealed by widespread effects of *dac* depletion through RNAi. The activity of *dac* is required for normal growth and joint formation in the funicle (Fig. 5B) and to impart funicle identity in articles 7–8, which adopt a club-like identity in *dac* RNAi resultants (Fig. 5C). These phenotypes are consistent with the overwhelming bias in *dac* expression toward the funicle in *T. castaneum* (Fig. 3B). In the legs of both *T. castaneum* (pers. obs.) and *D. melanogaster* (Mardon et al., 1994), *dac* is required for growth and patterning of a large intermediate domain. Our data suggest that this broader domain of function may have been present in the antennae of ancestral holometabolous insects as well, in which case the broad but largely non-functional expression domain found in *D. melanogaster* may be a remnant of this ancestral domain that has lost interactions with target genes as this lineage evolved away from ancestral morphology.

Several genes examined in this study have functions in *T. castaneum* that closely resemble the developmental function their orthologues perform in *D. melanogaster*. For example, in both species, *ss* activity is required for antennal identity, and reduction in *ss* leads to antenna-to-leg transformations. However, neither *Dll* nor *hth* RNAi caused transformation of the antenna to leg in *T. castaneum*; such transformations would be expected given the *Drosophila* model, in which *Dll* and *hth* overlap extensively in expression in the antenna where they cooperatively activate *ss* (Dong et al., 2000; Emmons et al., 2007). An evolutionarily ancient role for *hth* and its cofactor *extra-denticle* (*exd*) in the specification of antennal identity is supported by the partial antenna-to-leg transformations that occur in response to depletion of either *hth* or *exd* through RNAi in the orthopteran *Gryllus bimaculatus* (Mito et al., 2008; Ronco et al., 2008), although *hth* and *Dll* expressions have only a narrow ring of overlap in orthopteran antennae (Jockusch et al. 2004; Ronco et al. 2008). One possible interpretation of the *T. castaneum* data is that *hth* and *Dll* are required during embryonic specification of appendage identity, but that *ss* activity has become independent of these inputs by metamorphosis. Consistent with this, *hth* is required only transiently during the first or early second instar in *D. melanogaster*; however, *Dll* activity is required continuously through at least the third instar in *Drosophila* (Emmons et al., 2007). This result may also be due to residual gene activity in RNAi resultants; however, currently no evidence supports a role for *Dll* or *hth* in specifying antennal identity in *T. castaneum*. Depletion of *Dll* using RNAi in the milkweed bug *Oncopeltus fasciatus* also did not produce antenna-to-leg transformations (Angelini and Kaufman, 2004), raising the possibility that the role of *Dll* in the determination of antennal identity is a derived role in flies.

Although we do not have evidence for a conserved role for *Dll* in the determination of antennal identity, it does share a role in PD axis elongation in the antennae of both *T. castaneum* and *D. melanogaster*. A *Dll* target in *D. melanogaster*, the EGF pathway, also has a shared role in distal axis elongation in both species. Other genes have functions that are homologous only at the level of patterning, but not at the level of specific structures. For example, *al* acts to pattern the distal structures in both species, despite drastic differences in the morphology of those structures (the arista in *Drosophila* and the distal club articles in *Tribolium*), but it does not appear to be regulated by *Lim1* in *T. castaneum*, as it is in *D. melanogaster* (Campbell, 2005). By degrees, some genes have functions that are less similar in the two species, like *rn* and *pdm*, which pattern areas at roughly corresponding levels of the PD axis, but with greater or smaller domains without identifiably homologous limits.

In *Drosophila*, Notch pathway activity appears to function similarly in leg and antennal development (Rauskolb and Irvine, 1999). Elimination of Notch signaling activity (by reduction in expression of *Notch* or of its ligands) leads to decreased appendage growth along the PD axis and loss of joints, while ectopic expression promotes growth and joint formation (Bishop et al., 1999; Rauskolb and Irvine, 1999). Similarly, depletion of *Ser* in *T. castaneum* resulted in a severe reduction in growth and in loss of joints, showing that both functions are conserved for *Ser*. By contrast, RNAi against *Notch* lead to an almost complete loss of joints but did not cause substantial reductions in antennal growth in *T. castaneum*. As Notch is the only known receptor for the Ser ligand, the most plausible explanation for the greater severity of *Ser* RNAi phenotypes is that we are only able to observe weak *Notch* knockdown phenotypes, with greater reduction in *Notch* expression leading to lethality. Consistent with this, survival was low after *Notch* depletion (Table 2). *Notch* also has a conserved role in sensory bristle development. *Notch* RNAi resultants lacked sensory bristles (but not macrochetes) in *T. castaneum*. By contrast, *Ser*-depleted phenotypes retained both the macrochetes and smaller sensory hairs. In *D. melanogaster*, bristle development is dependent on Notch signaling, and can be eliminated either by reduction in *Notch* expression (Heitzler and Simpson, 1991) or by simultaneous reduction in both *Ser* and *Delta*, which encodes the second Notch ligand, but not by reduction of either ligand on its own (Zeng et al., 1998).

A general inter-relatedness of appendage growth and joint formation was observed for many gene depletions affecting the funicle. As the funicle was reduced in length, the number of articles was decreased, and the remaining articles were similar in size and shape to wildtype articles (e.g., Figs. 4B, F, 5D–I, 6E, F). A similar pattern is observed in *D. melanogaster* mutants. However, several *D. melanogaster* mutants affecting only one of these processes have been identified, suggesting a model in which a common upstream factor (such as Notch signaling) activates independent downstream pathways, one regulating growth and another regulating joint formation (de Celis et al. 1998). Our observation that *Notch* depletion can affect joint formation independently of axis elongation is also consistent with this model, as is the observation that the funicle of some *ss* RNAi resultants is of normal length but lacks some joints. Regulation of growth may be different in the club, as club segments

never appeared entirely deleted, while such deletions were common in the funicle.

Taken together, it is interesting to consider that genes with conserved functions are those involved in developmental events that are also conserved, such as outgrowth of the proximal-distal axis or joint formation. Events such as establishment, growth, and segmentation of regions within the flagellum are distinct developmental processes in *Drosophila* and *Tribolium*, and our data demonstrate that the functions of genes governing these events differ extensively between these species.

A developmental model of patterning in the beetle antenna

Two previous models have been proposed to describe patterning of the beetle antenna. Dawson (1966) suggested a model based on the frequency of article fusions in various antenna patterning mutants in *T. castaneum*. More recently, Minelli (2005) proposed a model of beetle antennal ontogeny based on comparisons to the development of antennae in several insect and crustacean groups. These models differ in their details, but both describe the progressive differentiation of sequentially smaller domains in the developing adult flagellum.

The results presented here are consistent with both proposals, and these data lead us to a genetic model of antennal ontogeny (Fig. 7). (1) The initial segmentation of the antenna occurs during embryogenesis. However, it is not yet clear how the identity of these segments corresponds to the identity of adult segments in *T. castaneum*. (2) During the prepupal stage (early metamorphosis) *ss* is necessary for the maintenance of antenna identity, and while it is likely *ss* also has this function in the embryo, it remains to be tested. (3) Several factors are required for growth of the flagellum at metamorphosis (e.g., EGF, *Ser*, and *Dll*). (4) Differentiation of the club and funicle then occurs. The activity of *dac* is required in the funicle to inhibit club identity in more distal cells. A gene promoting club identity has not been identified. (5) Genes including *odd*-related paralogues, *dac*, and *hth* promote growth within the funicle. Other currently unknown genes likely perform a similar role in the club. Other genes promote growth in progressively more local areas. (6) At least in the club, specific genes are required to promote the development of particular joints. (7) Joint formation is activated through Notch signaling via the Serrate ligand. These steps have been presented in a logical, conceptual order, but more work will be necessary to establish the timing of these events.

Conclusions

Based on an understanding of antenna development provided by studies of *Drosophila* it was not clear how the capitate antenna of *T. castaneum* would be regionalized. By screening a large number of candidate developmental patterning genes using RNAi, we were able to characterize patterning in the adult antenna of *T. castaneum*. A small number of genes were conserved in function between species, while many genes appear to perform distinct roles in each species (Fig. 2). The maintenance of antenna identity in *Tribolium* requires *ss*, as in *Drosophila*. Joint formation between articles requires *Notch* and *Serrate*, which has also been shown for *D. melanogaster*. However regionalization of the flagellum involves gene activities unlike those of *Drosophila* orthologues. Growth of the funicle requires *odd* paralogues, *hth*, and *dac*. *dac* also acts to direct funicle identity. *pdm*, *al*, *ap*, and *bab* contribute to joint formation in the club.

It is likely that gene functions and developmental processes that are similar between these two insects are indicative of processes inherited from their common ancestor. Therefore the developmental features shared by *Drosophila* and *Tribolium* are likely to be found in many other holometabolous insects. However, in the absence of data from additional species, it is not possible to determine the direction of evolutionary change for genes with different functions. The developmental genetic model presented here from *T. castaneum* provides a starting point for a mechanistic understanding of the morphological diversity of antennae of beetles and other insects. For example, species in which the funicle-club boundary lies between articles 6 and 7 (e.g. *Tribolium confusum*) could be explained by a shift in the expression of one or more genes controlling funicle or club development, or by an absence of homeotic activity in *dac*. Despite the highly conserved number of antennal articles in Coleoptera (Minelli, 2005), the number and expanse of flagellar regions varies greatly among the antennae of beetles. The flagellar regionalization of some beetles is very elaborate, with four or more distinct identities (e.g. some scarabs and silphids) or with articles of similar identity that are not adjacent (e.g. *Aristobia approximator* and *Anisotoma* spp.). Elucidation of the developmental basis of these morphologies must await further study in *Tribolium* and other coleopteran species.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version at, doi:10.1016/j.ydbio.2008.10.047.

References

Angelini, D.R., Kaufman, T.C., 2004. Functional analyses in the hemipteran *Oncopeltus fasciatus* reveal conserved and derived aspects of appendage patterning in insects. Dev. Biol. 271 (2), 306–321.

Angelini, D.R., Kaufman, T.C., 2005. Insect appendages and comparative ontogenetics. Dev. Biol. 286 (1), 57–77.

Beermann, A., Jay, D.G., Beeman, R.W., Hülskamp, M., Tautz, D., Jürgens, G., 2001. The *Short antennae* gene of *Tribolium* is required for limb development and encodes the orthologue of the *Drosophila* Distal-less protein. Development 128, 287–297.

Bishop, S.A., Klein, T., Arias, A.M., Couso, J.P., 1999. Composite signalling from *Serrate* and *Delta* establishes leg segments in *Drosophila* through Notch. Development 126 (13), 2993–3003.

Campbell, G., 2005. Regulation of gene expression in the distal region of the *Drosophila* leg by the *Hox11* homolog, *C15*. Dev. Biol. 278 (2), 607–618.

Campbell, G., Tomlinson, A., 1998. The role of the homeobox genes *aristaless* and *Distal-less* in patterning the legs and wings of *Drosophila*. Development 125 (22), 4483–4493.

Casares, F., Mann, R.S., 2001. The ground state of the ventral appendage in *Drosophila*. Science 293, 1477–1480.

Chapman, R.F., 1998. Chordotonal organs. The Insects: Structure and Function. Cambridge University Press, Cambridge, pp. 617–629.

Chu, J., Dong, P.D.S., Panganiban, G., 2002. Limb type-specific regulation of *bric a brac* contributes to morphological diversity. Development 129 (3), 695–704.

Cohen, S.M., Jürgens, G., 1989. Proximal-distal pattern formation in *Drosophila*: graded requirement for *Distal-less* gene activity during limb development. Roux's Arch. Dev. Biol. 198, 157–169.

Couderc, J.-L., Godt, D., Zollman, S., Chen, J., Li, M., Tiong, S., Cramton, S.E., Sahut-Barnola, I., Laski, F.A., 2002. The *bric à brac* locus consists of two paralogous genes encoding BTB/POZ domain proteins and acts as a homeotic and morphogenetic regulator of imaginal development in *Drosophila*. Development 129, 2419–2433.

Dawson, P.S., 1966. Insect antennal development and the *serrate* gene in *Tribolium castaneum*. J. Theor. Biol. 12 (1), 133–139.

de Celis Ibeas, J.M., Bray, S.J., 2003. Bowl is required downstream of Notch for elaboration of distal limb patterning. Development 130, 5943–5952.

de Celis, J.F., Tyler, D.M., de Celis, J., Bray, S.J., 1998. Notch signalling mediates segmentation of the *Drosophila* leg. Development 125 (23), 4617–4626.

Díaz-Benjumea, F.J., Cohen, B., Cohen, S.M., 1994. Cell interaction between compartments establishes the proximal-distal axis of *Drosophila* legs. Nature 372 (6502), 175–179.

Dong, P.D., Chu, J., Panganiban, G., 2000. Coexpression of the homeobox genes *Distal-less* and *homothorax* determines *Drosophila* antennal identity. Development 127 (2), 209–216.

Dong, P.D., Dicks, J.S., Panganiban, G., 2002. *Distal-less* and *homothorax* regulate multiple targets to pattern the *Drosophila* antenna. Development 129 (8), 1967–1974.

Duncan, D.M., Burgess, E.A., Duncan, I., 1998. Control of distal antennal identity and tarsal development in *Drosophila* by *spineless-aristapedia*, a homolog of the mammalian dioxin receptor. Genes Dev. 12 (9), 1290–1303.

Emerald, B.S., Curtiss, J., Mlodzik, M., Cohen, S.M., 2003. *distal antenna* and *distal antenna related* encode nuclear proteins containing pipsqueak motifs involved in antenna development in *Drosophila*. Development 130 (6), 1171–1180.

Emmons, R.B., Duncan, D., Duncan, I., 2007. Regulation of the *Drosophila* distal antennal determinant *spineless*. Dev. Biol. 302 (2), 412–426.

Galindo, M.I., Bishop, S.A., Greig, S., Couso, J.P., 2002. Leg patterning driven by proximal-distal interactions and EGFR signaling. Science 297 (5579), 256–259.

Godt, D., Couderc, J.L., Cramton, S.E., Laski, F.A., 1993. Pattern formation in the limbs of *Drosophila*: *bric à brac* is expressed in both a gradient and a wave-like pattern and is required for specification and proper segmentation of the tarsus. Development 119 (3), 799–812.

Heitzler, P., Simpson, P., 1991. The choice of cell fate in the epidermis of *Drosophila*. Cell 64 (6), 1083–1092.

Herbst, J.F.W., 1797. Der Käfer. In: Jablonsky, C.G., Herbst, J.F.W. (Eds.), Natursystem aller bekannten in-und ausländischen Insecten, als eine Fortzetsung der von Büffonschen Naturgeschichte. J. Pauli, Berlin, p. 346.

Jockusch, E.L., Williams, T.A., Nagy, L.M., 2004. The evolution of patterning of serially homologous appendages in insects. Dev. Genes Evol. 214, 324–338.

Kojima, T., 2004. The mechanism of *Drosophila* leg development along the proximo-distal axis. Dev. Growth Differ. 46, 115–129.

Kulkarni, M.M., Booker, M., Silver, S.J., Friedman, A., Hong, P., Perrimon, N., Mathey-Prevot, B., 2006. Evidence of off-target effects associated with long dsRNAs in *Drosophila melanogaster* cell-based assays. Nature Methods 3, 833–838.

Lecuit, T., Cohen, S.M., 1997. Proximal-distal axis formation in the *Drosophila* leg. Nature 388 (6638), 139–145.

Mardon, G., Solomon, N.M., Rubin, G.M., 1994. *dachshund* encodes a nuclear protein required for normal eye and leg development in *Drosophila*. Development 120, 3473–3486.

Minelli, A., 2005. A segmental analysis of the beetle antenna. Studi Trentini Acta Biologica 81 (2004), 91–101.

Mito, T., Ronco, M., Uda, T., Nakamura, T., Ohuchi, H., Noji, S., 2008. Divergent and conserved roles of *extradenticle* in body segmentation and appendage formation, respectively, in the cricket *Gryllus bimaculatus*. Dev. Biol. 313 (1), 67–79.

Ober, K.A., Jockusch, E.L., 2006. The roles of *wingless* and *decapentaplegic* in axis and appendage development in the red flour beetle, *Tribolium castaneum*. Dev. Biol. 294 (2), 391–405.

Pfaffl, M.W., 2001. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. 29 (9), e45.

Prpic, N.M., Wigand, B., Damen, W.G., Klingler, M., 2001. Expression of *dachshund* in wild-type and *Distal-less* mutant *Tribolium* corroborates serial homologies in insect appendages. Dev. Genes Evol. 211 (10), 467–477.

Rauskolb, C., 2001. The establishment of segmentation in the *Drosophila* leg. Development 128 (22), 4511–4521.

Rauskolb, C., Irvine, K.D., 1999. Notch-mediated segmentation and growth control of the *Drosophila* leg. Dev. Biol. 210 (2), 339–350.

Ronco, M., Uda, T., Mito, T., Minelli, A., Noji, S., Klingler, M., 2008. Antenna and all gnathal appendages are similarly transformed by *homothorax* knock-down in the cricket *Gryllus bimaculatus*. Dev. Biol. 313 (1), 80–92.

Schoppmeier, M., Damen, W.G., 2001. Double-stranded RNA interference in the spider *Cupiennius salei*: the role of *Distal-less* is evolutionarily conserved in arthropod appendage formation. Dev. Genes Evol. 211 (2), 76–82.

Snodgrass, R.E., 1928. Morphology and evolution of the insect head and its appendages. Smithson. Inst. Misc. Collect. 81 (3), 1–158.

Snodgrass, R.E., 1935. Principles of Insect Morphology. McGraw-Hill, New York.

Sokoloff, A., 1972. The Biology of *Tribolium*. Vol 1. Clarendon Press, Oxford.

Švácha, P., 1992. What are and what are not imaginal discs: reevaluation of some basic concepts (Insecta, Holometabola). Dev. Biol. 154 (1), 101–117.

Tomoyasu, Y., Denell, R.E., 2004. Larval RNAi in *Tribolium* (Coleoptera) for analyzing adult development. Dev. Genes Evol. 214, 575–578.

Tomoyasu, Y., Wheeler, S.R., Denell, R.E., 2005. *Ultrabithorax* is required for membranous wing identity in the beetle *Tribolium castaneum*. Nature 433 (7026), 643–647.

Tribolium Genome Sequencing Consortium, 2008. The genome of the model beetle and pest *Tribolium castaneum*. Nature 452, 949–955.

Tsuji, T., Sato, A., Hiratani, I., Taira, M., Saigo, K., Kojima, T., 2000. Requirements of *Lim1*, a *Drosophila* LIM-homeobox gene, for normal leg and antennal development. Development 127, 4315–4323.

Van der Meer, J.M., 1977. Optical clean and permanent whole mount preparation for phase-contrast microscopy of cuticular structures of insect larvae. Drosoph. Inf. Serv. 52, 160.

Wu, J., Cohen, S.M., 1999. Proximodistal axis formation in the *Drosophila* leg: subdivision into proximal and distal domains by Homothorax and Distal-less. Development 126 (1), 109–117.

Zeng, C., Younger-Shepherd, S., Jan, L.Y., Jan, Y.N., 1998. Delta and Serrate are redundant Notch ligands required for asymmetric cell divisions within the *Drosophila* sensory organ lineage. Genes Dev. 12 (8), 1086–1091.